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Research Article

# miR-125b targets DNMT3b and mediates p53 DNA methylation involving in the vascular smooth muscle cells proliferation induced by homocysteine

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ABSTRACT

MicroRNAs (miRNAs) are short non-coding RNA and play crucial roles in a wide array of biological processes, including cell proliferation, differentiation and apoptosis. Our previous studies found that homocysteine(Hcy) can stimulate the proliferation of vascular smooth muscle cells (VSMCs), however, the underlying mechanisms were not fully elucidated. Here, we found proliferation of VSMCs induced by Hcy was of correspondence to the miR-125b expression reduced both *in vitro* and in the ApoE knockout mice, the hypermethylation of p53, its decreased expression, and DNA (cytosine-5)-methyltransferase 3b (DNMT3b) up-regulated. And, we found DNMT3b is a target of miR-125b, which was verified by the Dual-Luciferase reporter assay and western blotting. Besides, the siRNA interference for DNMT3b significantly decreased the methylation level of p53, which unveiled the causative role of DNMT3b in p53 hypermethylation. miR-125b transfection further confirmed its regulative roles on p53 gene methylation status and the VSMCs proliferation. Our data suggested that a miR-125b-DNMT3b-p53 signal pathway may exist in the VSMCs proliferation induced by Hcy.



## 1. Introduction

Experimental and clinical studies have established that Homocysteine (Hcy) is an independent risk factor for atherosclerosis (AS) b [1,2]. Both our studies and other researches have reported that Hcy can stimulate the proliferation of vascular smooth muscle cells (VSMCs) [3–5], which is the primary kinds of cells in vascular wall. The proliferation of VSMCs is an essential fact in AS plaque, which is deeply involved in the pathogenesis of AS. Lots of mechanisms have been proposed to explain the genesis of VSMCs proliferation in order to target a potential gene for its reversal [6–8], but the underlying mechanisms remain not fully understood.

MicroRNAs (miRNAs) are small and noncoding RNA molecules of approximately 22 nucleotides that bind to their target mRNAs and inhibit translation, which play crucial roles in eukaryotic gene regulation, especially in cell proliferation, apoptosis, and differentiation [9–11]. Generally, miRNAs can be perfect or imperfect complementarily bind to the mRNAs' 3′untranslated region (3′UTR) of the target genes, which result in mRNA degradation or translational repression [12,13]. Recently, more and more studies have demonstrated that miRNAs dysregulation are associated with cardiovascular diseases, including cardiac hypertrophy, heart failure, and vascular atherosclerosis [14,15]. For example, miR-21, miR-143, miR-145 [16–18] have been suggested to play pivotal roles in VSMCs proliferation as an essential regulator and involved in various aspects of cardiovascular diseases. In addition, some miRNAs were suggested to be involved VSMCs proliferation by targeting specific genes and via signal pathways. miR-26a inhibited VSMCs differentiation and apoptosis while promoting proliferation through a mechanism that targets the transforming growth factor-β–bone morphogenetic protein pathway [19].

*Abbreviations:* VSMCs, Vascular Smooth Muscle Cells; AS, atherosclerosis; Hcy, homocysteine; HHcy, Hyperhomocysteinemia; DNMT3b, DNA (cytosine-5)-methyltransferase 3b; nMS-PCR, nested methylation-specific-PCR; UTR, untranslated region; miRNAs, microRNAs; mfe, minimum free energy

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miR-146a and Krüppel-like factor 4 constitute a feedback loop to regulate VSMC proliferation and vascular neointimal hyperplasia [20]. miR-125b, a human homologue of lin-4, is reported that it is involved in vascular calcification *in vitro* and *in vivo*, at least partially by targeting SP7 [21]. And Louisa et al. [22] found that elevated levels of miRNA-125b in VSMCs of diabetic db/db mice caused increased inflammatory gene expression by targeting the histone methyltransferase Suv39h1 in diabetes. These researches suggested that miR-125b involves in regulation of VSMCs and plays a crucial role in the crosstalk between other epigenetics such as histone methylation. However, the role of miR-125b in the proliferation of VSMCs and with DNA methylation remains unkown.

It is well-known that p53 is a key tumor suppressor and a master regulator of various signaling pathways, including cell-cycle regulation, induction of apoptosis, development and differentiation [23]. p53 functions largely as a transcription factor, which can trigger a variety of anti-proliferative programs by activating or repressing key effects or genes [24]. For VSMCs, p53 down-regulation precedes vascular smooth muscle cells migration and proliferation [25]. For the regulation of p53, despite a significant body of literatures deal with this puzzle, much remains poorly understood. Le et al. [26] recently have demonstrated an important role of miRNAs on the regulation of p53, the authors found that miR-125b was an important negative regulator for p53; miR-125b mediated a down-regulation of p53 by binding to a miRNA response element in the 3′UTR of p53 mRNA. Schroeder et al. [27] found the methylation-dependent regulation of p53; they provided the first experimental evidence for DNA methylation dependent silencing of the p53 gene promoter. Also, studies recently have reported that miRNAs can be involved in the promoter methylation of CpG islands by targeting DNMTs' 3'UTR [28,29]. And, miRNAs and DNA methylation are the essential modes for epigenetic regulation of genes; the question is: Could the Hcy-stimulated VSMCs proliferation go through the miRNAs mediated methylation regulated by DNMTs on modification of p53 gene and thus regulate its expression?

In this study, we investigated the expression and DNA methylation status of p53, the expression of miR-125b, using its precursor and inhibitor to testify miR-125b targets DNMT3b via Dual-Luciferase Reporter Assay; we also detected the changes of DNA methylation levels in the promoter of p53 and its expression after transfected by DNMT3b siRNA. We found that a miR-125b-DNMT3b-p53 signal pathway may exist in the VSMCs proliferation induced by Hcy. Unveiling the details of these pathways may provide a novel strategy for a reversal of Hcy-mediated VSMCs proliferation in AS.

## 2. Material and methods

### 2.1. Cell culture

Primary culture of VSMCs was obtained from the media of the umbilical vein of Homo sapien [30]. Then the cells were identified by immunocytochemistry using the anti-α-actin antibody. When VSMCs grew to 80% confluence, serum was deprived for 24 h to reach synchronization, and then cells were cultured with 5% fetal calf serum for another 24 h before DL-Homocysteine (Sigma, USA) addition. Hcy was applied at the concentrations of 0 (control), 50, 100, 200, 500 μM and 100 μM Hcy + 30 μM folate + 15 μM $VitB_{12}$(Sigma, USA)(Folate group) respectively. In order to compensate for the short half-life of Hcy, it was replenished every 8 h (total three times).

### 2.2. Animals

The atherosclerosis mice model was constructed with methionine diet as described previously [31]. Male wild-type mice (C57BL/6 J) and ApoE knockout ($ApoE^{-/-}$) mice on a C57BL/6 J genetic background performed in the experiments were purchased from the Department of Laboratory Animal Science of the Beijing University Health Science Center (Beijing, China). The treatment of the laboratory animals and experimental protocol followed the guidelines of General Hospital of Ningxia Medical University that was approved by the Institutional Authority for Laboratory Animal Care. The mice were divided randomly into three groups (n = 6 each group) and feeding on the following diets (KeAoXieLi, Beijing, China): (1) Control group: $ApoE^{-/-}$ mice fed with regular diet; (2) ApoE-Meth group: $ApoE^{-/-}$ mice fed with regular diet plus 1.7% methionine (wt/wt) and (3) Wild-Meth group: wild-type mice fed with regular diet plus 1.7% methionine (wt/wt); After 15 weeks of feeding, the mice were sacrificed, and the tissues were collected and stored at −80 °C for further analysis.

### 2.3. Quantitative real-time PCR(qPCR)

The total RNA was isolated by applying TRIZOL reagent (Invitrogen, USA). And the RNA was reversely transcribed by the Revert Aid first strand cDNA synthesis kit (Fermentas, USA). The primer nucleotide sequences: DNMT3b forward primer: 5′-AGCTCTTACCTTACCATC-3′; reverse primer: 5′-CCAT CCTGATACTCTGAA-3′; p53 forward primer: 5′-CAGCATCTTATC CGAGTG-3′; reverse primer: 5′-GATGGTGGTACAGT CAGA-3′. And the target sequences for miR-125b were: GGG TCCCTGAGACCC TAACTTGTGA and GCTGTC AAC ATACGCTACG TAACG. The real-time PCR was carried out by applying an FTC-3000 real-time PCR detection system (Funglyn Biotech, Canada) with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control for DNMT3b and p53 and U6 for miR-125b detection. Each Real-Time PCR assay was performed in triplicate and included positive, negative and no template reagent controls.

### 2.4. Western blotting

Whole-cell proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) according to the manufacturer's instructions. 20 μg of whole-cell proteins were separated by 8% SDS-PAGE, then the proteins were then transferred onto an Immobilon-P transfer polyvinylidene fluoride membrane (PDVF, pore size: 0.45 μm; Millipore, USA) with a Semi-dry Transfer Cell (Model 755, Bio-Rad, USA) for 90 min, allowing the pre-stained marker to be completely transferred from the gel to the membrane. The gel was discarded and the membrane was incubated at room temperature in 5% non-fat milk prepared with PBS-T buffer. 2 h later, the membrane was incubated with primary antibody of anti-DNMT3b (abcam USA, http://www.abcam.com/dnmt3b-antibody-chip-grade-ab2851.html), anti-p53 (http://www.abcam.com/p53-antibody-ab31333.html) at 4 °C overnight. The membrane was washed three times with PBS-T buffer and the secondary antibody was added for 2 h at room temperature. The membrane was washed three times with TBS-T buffer and developed using ECL reagents (Thermo Scientific Pierce, USA).

### 2.5. shRNA for DNMT3b knockdown

Three vectors with shRNA expressed siRNA for DNMT3b were purchased from Genechem (ShangHai, China). The sense sequences are 5′-AGGACCAGCTGAAGCCCATT-3′, 5′-GGAAAGAT CAAGGGCTTCTT-3′, 5′-TCGTGTGGGGA AAGATCAAT-3′. A negative